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ANALYTICAL STUDIES OF MORPHINE AND RELATED SUBSTANCES USING LC-MS/MS

Maria Andersson

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Analytical studies of morphine and related substances using LC-MS/MS

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

Maria Andersson

Principal Supervisor:
Olof Beck
Karolinska Institute
Department of Laboratory medicine
Division of Clinical Pharmacology

Co-supervisor(s):
Linda Björkhem-Bergman
Karolinska Institute
Department of Laboratory medicine
Division of Clinical Microbiology

Inger Öhman
Karolinska Institute
Department of Medicine, Solna
Division of Center for Pharmacoepidemiology

Opponent:
Markus Meyer
Saarland University
Department of Experimental and Clinical Toxicology
Division of Experimental and Clinical Pharmacology and Toxicology

Examination Board:
Eva Vikström Johnsson
Karolinska Institute
Department of Medicine, Solna
Division of Clinical Pharmacology

Mikael Hedeland
Uppsala University
Department of Medicinal Chemistry
Division of Analytical Pharmaceutical Chemistry

Jan Kehr
Karolinska Institute
Department of Physiology and Pharmacology

ABSTRACT

Morphine is considered to be metabolized in three distinct metabolic pathways; glucuronidation, sulfation and N-demetylation. However, identification of morphine-3-sulfate (M3S) and morphine-6-sulfate (M6S) as morphine metabolites has not been convincing according to previous literature due to lack of reliable reference material and identification based on thin layer chromatography. In this thesis reference material for M3S and M6S was developed, and a sensitive analytical method for quantification of M3S and M6S in urine and plasma with mass spectrometry was also developed. Urine and plasma were analysed from different study groups; newborns, heroin addicts and terminal cancer patients. M3S was present in both urine and plasma from all study groups. The plasma ratio M3S/morphine-3-glucuronide was found to be 30 times higher in newborns than in adults. There was weak evidence that M6S actually forms *in-vivo* since only two samples contained detectable concentrations of M6S. It was demonstrated that both M3S and M6S was formed *in-vitro* by human liver homogenate but in small amounts. Nevertheless, we have demonstrated that both M3S and M6S are morphine metabolites in humans.

Heroin is a highly addictive morphine derivative that is present on the illicit drug market. One of the primary interests in clinical and forensic drug testing is determination/identification of heroin intake. In this thesis a new validated routine LC-MS/MS method for urine drug testing of opiates has been evaluated leading to increased selectivity and separation power compared to earlier GC-MS methods. The evaluation displayed that the 6-AM biomarker is a good and dependable criterion for a heroin intake. In addition, we have also demonstrated that this method can be reduced regarding number of analytes.

In 11.5 % of 6-AM positive urine samples (n=693) an atypical metabolic pattern of morphine and 6-AM was observed after a heroin intake. The atypical pattern seemed not to be related to a genetic polymorphism in the enzymes involved since the same individual can produce both “normal” and atypical pattern. *In-vitro* study using liver homogenates revealed that a strong inhibition of 6-AM formation was seen for a rearrangement product of thebaine (compound 3).

Indeed, this compound could also be identified in all patient samples showing the atypical pattern.

LIST OF SCIENTIFIC PAPERS

I. **Andersson M**, Janosik T, Shirani H, Slätt J, Fischer A, Beck O. Synthesis and bioanalytical evaluation of morphine-3-*O*-sulfate and morphine-6-*O*-sulfate in human urine and plasma using LC-MS/MS.
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II. **Andersson M**, Stephanson N, Öhman I, Terzuoli T, Beck O. A direct and efficient liquid chromatographic-mass spectrometric method for opiates in urine drug testing – importance of 6-acetylmorphine and reduction of analytes. Drug Test Anal. 2014 Apr;6(4):317-24

III. **Andersson M**, Björkhem-Bergman L, Ekström L, Bergqvist L, Lagercrantz H, Rane A, Beck O. On the presence of morphine-3-sulfate and morphine-6-sulfate in human urine and plasma, and formation in liver cytosol. *Submitted*

IV. **Andersson M**, Björkhem-Bergman L, Beck O. Studies on the inhibition of heroin metabolism. *In manuscript*

CONTENTS

1 Background 1
1.1 Opium and morphine 1
1.2 Heroin 1
1.3 Human carboxylesterase 4
1.4 Morphine metabolism 4
1.4.1 Sulfotransferases 6
1.5 Urine drug testing 7
1.5.1 Immunoassay 9
1.5.2 GC-MS 10
1.5.3 From HPLC to LC-MS/MS 10
2 Aims 14
3 Methods 15
3.1 Clinical samples 15
3.1.1 Study I 15
3.1.2 Study II 15
3.1.3 Study III 15
3.1.4 Study IV 16
3.2 liver tissue and cytosol 16
3.2.1 Study III and IV 16
3.3 sample preparation procedures 16
3.3.1 Plasma; Study I and III 16
3.3.2 Urine; Study I-IV 16
3.3.3 Liver cytosol; Study III 17
3.3.4 Liver homogenate; Study IV 17
3.4 bioanalysis 17

3.4.1 LC-MS/MS Study I-IV 17
3.4.2 LC-HRMS 18
3.4.3 CEDIA immunoassay for opiates 18
3.4.4 DRI Ethyl Glucuronide and Ethyl Alcohol 18
3.4.5 GC-MS for opiates 19
4 Results 20
4.1 Study I 20
4.1.1 Synthesis of M3S and M6S 20
4.1.2 Method development and validation 21
4.1.3 Application 23
4.2 study ii 24
4.2.1 Method design and validation 24
4.2.2 Interpretation strategy 27
4.2.3 Reduction of analytes 32
4.3 study III 34
4.3.1 Method development 34
4.3.2 Formation of M3S and M6S In Vitro 35
4.3.3 Presence of M3S and M6S *in-vivo* 36
4.4 study iv 38
4.4.1 Characterization of atypical samples 38
4.4.2 Study of 6-AM deacetylation *in-vitro* 39
4.4.3 Search for inhibitors in authentic samples 40
5 Discussion 42
6 Conclusion 45
7 Acknowledgements 46
8 References 48

LIST OF ABBREVIATIONS

3-AM	3-Acetylmorphine
6-AM	6-Acetylmorphine
APCI	Atmospheric pressure chemical ionisation
ASA	Acetyl salicylic acid
CEDIA	Cloned enzyme donor immunoassay
CES	Carboxyesterase
CG	Codeine-6-glucuronide
ESI	electrospray ionization
EtG	Ethyl glucuronide
EtOH	Ethyl alcohol
GC-MS	Gas chromatography mass spectrometery
LC-MS/MS	Liquid chromatography mass spectrometery
M3G	Morphine-3- glucuronide
M6G	Morphine-6- glucuronide
M3S	Morphine-3- sulfate
M6S	Morphine-6- sulfate
SA	Salicylic acid
PAPS	3'-phosphoadenosine-5' phosphosulfate phosphosulfate
UPLC	Ultra pressure liquid chromatography

1 BACKGROUND

1.1 OPIUM AND MORPHINE

Opium has been used throughout history as a medicinal plant. It is the condensed juice of unripe fruit capsules of the opium poppy, *Papaver somniferum*. The plant grows up to 1-1.5 meters in height with white, violet or purple flowers (1). It has been difficult to define where the plant originated but information points to the Mediterranean region of Asia Minor. Opium was mainly used for medical purposes due to its analgesic and sedative effects, but also as a recreational drug. Opium addiction was first described already in the year 1000 by Biruni, an Iranian physician. As the use and demand of opium increased the opium poppy began to be grown and processed in many countries (2).

Opium poppy contains a large number of alkaloids (1). Four of them have found medicinal use and are isolated from opium as natural products. Morphine is the main alkaloid (10-20 %) and the others are codeine (0.8-2.5 %), noscapine (4-8 %), and papaverine (~1 %). Morphine is relatively easy separated from the other alkaloids due to its phenolic properties (1). Morphine was first isolated 1817 from opium by the German apothecary Friedrich Sertürner who named it "morphium". A structure was first proposed 100 years later. In the end of the 19th century "morphium" was readily available and used for treatment of pain (3).

1.2 HEROIN

Heroin (3,6-diacetylmorphine, diamorphine) was introduced as a cough medicine 1898 by a German pharmaceutical company (Farbenfabriken vorm. Friedrich Bayer & Co., now Bayer AG) and was sold over the counter. Heroin is a highly addictive drug (4). And due to an epidemic misuse of heroin it was banned for medical use in the US in 1924. However, in the UK, heroin is still used as an analgesic drug (5).

Illicit heroin is produced from raw opium by acetylation with acetic acid anhydride and heat, leading to a chemically impure product. Impurities are

remains of opium alkaloids such as morphine, codeine, papaverine and noscapine, but illicit heroin also contain impurities as a result of the production process (6). Additional acetylated derivatives that are found in heroin are the *O*-acetylated acetylcodeine, 6-acetylmorphine and the *N*-acetylated acetylcodamine, acetylnarcotine and the rearrangements products compound 3 and 4 from thebaine (7, 8).

Heroin is also extensively mixed with adulterants and/or diluents in order to increase the amount of product (9). Some adulterants such as caffeine and procaine have a similar bitter taste as heroin (10). Seizures made in Denmark have shown continually shifting patterns of adulterants and diluents. In a study, the relative amount of 3,6-diacetylmorphine in different street heroin product seizures (n=146, during years 2002-2003) were between 3-51% with a mean content of 23%. Caffeine and paracetamol were the two most common. Other known adulterants are procaine, paracetamol, lead, strychnine (11) griseofulvin, diazepam, phenobarbital, piracetam, methaqualone, barbital, ascorbic acid, salicylic acid, mannitol, sucrose, glucose, lactos/maltose. (9).

Heroin is more lipophilic than morphine increasing its ability to pass the blood brain barrier. However, heroin is considered as a prodrug and that the pharmacological effect is accomplished by its metabolites, 6-acetylmorphine (6-AM) and morphine. The 3-acetyl moiety in heroin obstructs the binding to the stereospecific receptors resulting so that heroin displays low affinity to the opioid receptors. Conjugation at the 6-hydroxyl position does not prevent binding to the opioid receptor and hence such derivatives have pharmacological activity (12).

In humans, heroin is metabolized by liver carboxyesterases and serum pseudocholine esterases into 6-AM and further to morphine (Figure 1) (12). The conversion of heroin to 6-AM can also occur non-enzymatically (13, 14) Heroin has a short half-life in blood and is estimated to 5-7 min (15).

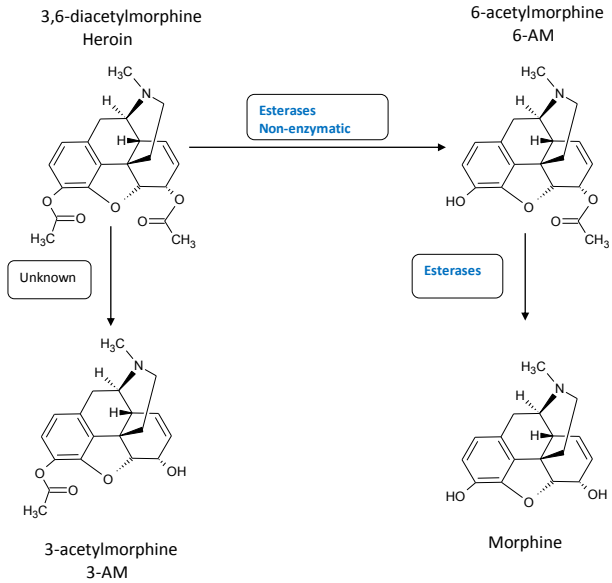


Figure 1 Heroin metabolism.

The intermediate 6-AM is formed almost instantly after a heroin intake and has a half-life around 20 min in plasma (16, 12).This leads to short window of detection (1-2 hours) of 6-AM in plasma. In urine, 6-AM remains longer leading to a slightly longer detection window of 2-8 hours (15).

Heroin is the drug most often implicated in drug overdoses with lethal outcome in Europe (17). It is estimated that there are 12-20 million heroin abusers (age 15-64 years) around the world (18). The risk of death is 20-30 times higher for a heroin addict as compared with a non-drug user (19). There are about 100 heroin related deaths in Sweden per year (20).

Heroin creates a state of euphoria, warmth and well-being, constriction of the pupils, nausea and respiratory depression. The respiratory depression is usually the direct cause of death after a heroin overdose. The continuous use of heroin is characterized by persistent cravings, development of tolerance, and dangerous

and painful withdrawal symptoms. The risk of drug/heroin overdoses are related to a number of factors such as poly drug, alcohol and benzodiazepine use. The purity of ingested heroin has also been discussed as a factor. Some investigations have concluded that the heroin purity has nothing to do with the heroin deaths while some publications have implied that the heroin deaths have been reduced when the street heroin purity has decreased (21). Another factor is a period of abstinence from heroin and factors related to individual health status (22).

1.3 HUMAN CARBOXYLESTERASE

The carboxylesterase (CES) enzymes are a family of phase I enzymes. There are three major human CES:s CES1, human CES2 (also known as the human intestine CES, hiCES) and human CES3. But also CES4 and CES7 occur in humans. Three major CES:s display wide variety of xenobiotics as substrates; acetyl salicylic acid, heroin, cocaine, metylphenidate and oseltamivir as well as endogenous esters and amides (23). CES1 is highly expressed in the liver but it has also in other tissues such as lung epithelia and heart. CES2 is present in the small intestine, such as kidney, liver, heart, brain. CES3 has been expressed in the liver and gastrointestinal tract in low amount compared to CES1 and CES2. No CES:s activity has been detected in blood of humans (24).

The conversion of heroin to 6-AM is considered only to be catalyzed by both CES1 and CES2 in the liver and by pseudocholinesterase in serum, as well as non-enzymatically. The formation of morphine from 6-AM is only catalyzed by CES2 (13, 14, 25), and CES2 is 1000 times more active than CES1 (25, 26).

1.4 MORPHINE METABOLISM

Morphine is naturally occurring in the (-) isomeric form (3). Morphine is considered to be metabolized in three distinct metabolic pathways regardless of route of administration: glucuronidation (60-70 %), sulfation (5-10 %) and N-demethylation (1-6 %) (3) (Figure 2). According to the review of Milne morphine-3-sulfate (M3S) constitutes 5 % of metabolites after a given dose of morphine (3). However, when carefully examining the literature the identification of M3S as a morphine metabolite is not convincing according to

present day standard due to lack of reference material and identification based on thin layer chromatography (TLC). In the early work of Yeh 1975 they did not conclude the presence of M3S, but in the later study from 1977 its presence is reported and the amount estimated to be about 1 % relative to M3G (27, 28)

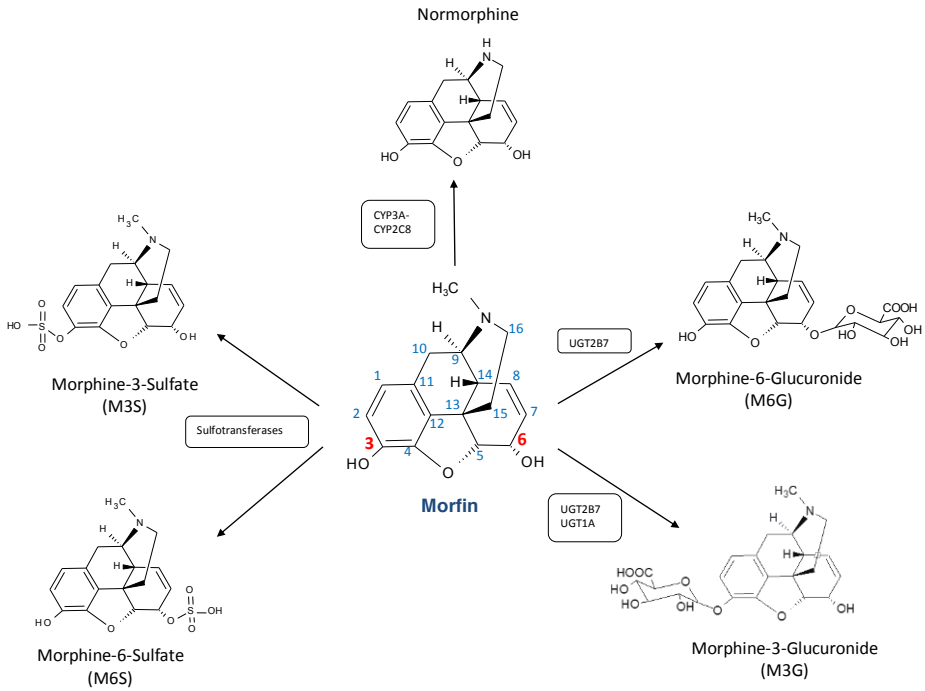


Figure 2 Morphine metabolism.

In a clinical study in preterm and newborn children M3S has been identified after an iv-dose of morphine by LC with UV detection. The M6S metabolite was not detected (29). Sulfation is an important metabolic pathway in fetal life, whereas glucuronidation becomes more important in adults (30). Hepatic glucuronidation in neonates has been described as immature at birth compared to the more mature neonatal hepatic sulfation. Some studies have demonstrated that neonates can significantly metabolize xenobiotics however, clearance is

considerable less compared to older infants and adults (31). The results obtained by Choonara suggested that morphine sulfation activity decreases with age (29).

Glucuronidation is an important clearance mechanism for many drugs and it is catalyzed by the enzymes UDP- glucuronosyl transferases (UGT) (32). The two hydroxyl groups of morphine differ in chemical nature. The hydroxyl at the 3-position is a phenol while the other hydroxyl group at the 6-position is a secondary allylic alcohol. The formation of M3G and M6G are both catalyzed by the UGT2B7 enzyme. The subenzyme UGT1A also contributes to the formation of M3G, but to a lesser extent. M3G does not bind to the opioid receptors and is not pharmacologically active (32). M6G has a high affinity to the opioid receptors leading to a greater analgesic effect than morphine itself (29). M6G has been suggested as a possible an alternative drug to morphine (3).

The N-demethylation of morphine to normorphine is catalyzed by cytocrom P450 (CYP) enzymes, mainly by CYP3A4 (~60 %) and CYP2C8 (~30 %) (33).

1.4.1 Sulfotransferases

Hepatic sulfation is a common phase II metabolic mechanism for increasing water solubility and decreasing biological activity. Sulfation is considered as a detoxification pathway. The sulfation reaction is catalyzed by sulfotransferases (SULTs) transferring the sulfonate ($SO3^-$) ion to a hydroxyl or amino function in the molecule (34, 35). The sulfonate transfer can be to different acceptor molecules. If the sulfonate group is transferred to an oxygen atom the reaction is called sulfation otherwise it is called sulfonation (36).

The membrane bound SULT enzymes catalyzes sulfation of peptides, proteins, lipids and carbohydrates. The cytosolic SULT enzymes catalyze the sulfation of xenobiotics and small endogenous compounds such as bile acids, steroids and neurotransmitters (35). SULT transfers a sulfonate group from 3'-phosphoadenosine-5' phosphosulfate (PAPS) (34). Sulfation is a phase II reaction, which often works in parallel with glucuronidation on the same substrates. It is not known which of these isoenzymes that is important for the morphine sulfation (34).

1.5 URINE DRUG TESTING

Detection of drugs in urine is a common laboratory investigation that has important clinical and forensic applications. The requirement is analytical methods that enable reliable and accurate identification and quantification of the parent drug and their metabolites in urine. The common strategy for urine drug testing is to perform two analytical investigations for a positive urine sample. The first investigation is made with an immunochemical screening method, which is fast, simple and relatively inexpensive, but less specific method. The second investigation is made on presumptive positives and is a confirmation method that is more selective, sensitive and more expensive. The methods for confirmation are often using mass spectrometry (37, 38). The combination of immunoassay as a screening and mass spectrometry as confirmation methods provides analytical results meeting forensic standards (38). In clinical toxicology for investigation of acute intoxication and in doping control mass spectrometry is often needed also in the screening analysis (39). High specificity and sensitivity is a requirement in clinical and forensic toxicology, and doping control due to the analytes are often not known and other endogenous compounds or xenobiotics may interfere the analysis (40).

The purpose of opiate drug testing is to determine if there is a drug intake. Since morphine is the target analyte in the screening one of the major tasks is therefore to determine which type opiate intake that has occurred. Heroin, morphine, codeine, ethylmorphine, opium and poppy seed intake can lead to presence of morphine in urine, see Table 1. It is therefore of importance to be able to differentiate the different possibilities by analyzing different biomarkers and their relative ratios (15). One way to determine a heroin intake has been using the morphine codeine ratio and another is to use 6-AM as a heroin biomarker. In some cases an atypical metabolic pattern of 6-AM relative to morphine has been observed (Figure 3) (41-45).

Table 1 Possible sources that can lead to morphine and other related analytes in urine are presented.

Intake	Analytes
Heroin	6-AM, morphine, M3G, M6G, codeine and CG
Codeine	Codeine, CG, morphine, M3G and M6G
Poppy seed	Morphine, M3G, M6G, codeine and CG
Morphine	Morphine, M3G and M6G

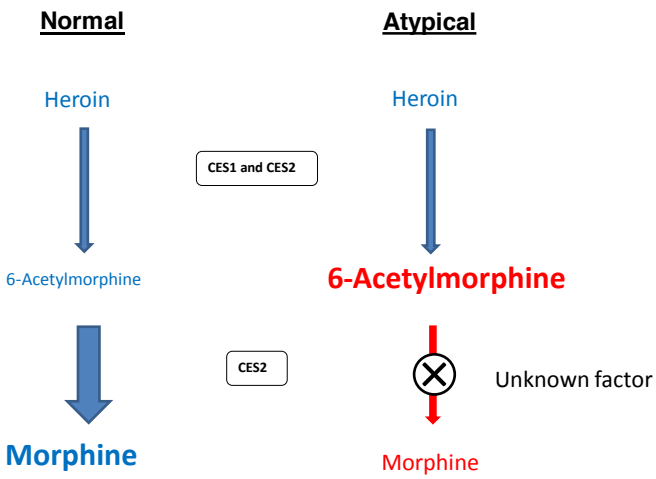


Figure 1 Simplified presentation of heroin metabolism showing the normal and atypical metabolic pattern. The first step from heroin to 6-acetylmorphine (6-AM) can be catalyzed by both CES1 and CES2 and by other esterases. The second conversion of 6-AM to morphine is mainly catalyzed by CES2.In the subjects showing an atypical pattern of heroin metabolism an unknown factor is inhibiting the second, enzymatic conversion from 6-AM to morphine.

During the last decade a development of less time-consuming and more selective analytical methods based on mass spectrometry has taken place (46). The golden standard is liquid or gas chromatography hyphenated to mass spectrometry for toxicology analyses. The demand for sensitivity and specificity are high due to the complex biological matrices which are attained with mass spectrometry (47).

Drug testing has traditionally been performed using urine samples. However, other biological matrices as oral fluid, breath, hair and blood can also be used. Different matrices have different detection times and should be chosen depending on the clinical requirement (47, 48).

1.5.1 Immunoassay

The most commonly used drug screening technique is immunoassay that first came in commercial use for drug testing in the 1970:s (38, 49). Different commercial tests are EIA (Enzymatic Immunoassay), EMIT (Enzyme Mediated Immunoassay Techniques), ELISA (Enzyme Linked Immunosorbent Assay), KIMS (Kinetic Interaction of Microparticles in Solution) and CEDIA (Cloned Enzyme Donor Immunoassay). They use the same basic principle; competition for binding to a selected drug binding antibody. If the analyte is present in the sample it will bind to the antibody (49). The binding of the antibody/analyte complex will lead to free tracer which is proportional to the drug concentration in the sample. The antibody binding site is not specific for a chemical substance but will show cross-reactivity to compounds with similar structure.

In the CEDIA assay for opiates the target analyte is morphine but the cross-reactivity for 6-AM and M3G is 81 % and for M6G 47 %. Cross-reactivity with non-opiate drugs is also occurring leading to false positive results. The CEDIA opiate screening gives 13 % false positives (48). The limited specificity makes immunoassays only suitable for qualitative screening and a more reliable and selective confirmation method is required for attaining accurate final results (38, 49).

1.5.2 GC-MS

In the 1980:s GC-MS became the method of choice in analytical toxicology which provided the requirement of the selectivity and sensitivity to detect and quantify the total morphine and total codeine concentrations. The sample preparation often consists of hydrolysis, extraction and derivatization (37, 50). Gas chromatography separates the urine samples components based on the components volatility and polarity. The separation of compounds occurs due to different retention times between the analytes. To acquire an accurate identification three characteristic ions are monitored (51). When hydrolysed conjugated morphine metabolites (3- and 6-morphineglucuronide and 3- and 6-morphine sulfate) as well as 6-AM will convert to morphine which result in the measurement of total morphine and codeine concentrations (37).

The GC-MS methods are safe and reliable but have some disadvantages such as need for time consuming sample preparation and relatively long run times. The confirmation with GC-MS also leads to lack of important information regarding the individual morphine metabolites.

1.5.3 From HPLC to LC-MS/MS

In high performance liquid chromatography (HPLC) analytes are being separated between a solid stationery phase and a mobile phase consisting of buffer and organic solvents. Often reversed phase chromatography is used which is when the stationary phase is lipophilic and the mobile phase is more hydrophilic (52).

The combination of liquid chromatography with mass spectrometry (LC-MS) has provided a technique with unique sensitivity and selectivity. The combination of these two technologies was based on the development of the electrospray interface. The difficulty in combining LC with MS is due to the liquid mobile phase that needs to vaporize in the ion source and enter the high vacuum MS system. The breakthrough arose when the interface of electrospray ionization (ESI) and atmospheric pressure chemical ionisation (APCI) was introduced (37, 53). In 2002 professor John Fenn, who invented the electrospray interface, was awarded the noble prize in chemistry for “development of

methods for identification and structure analyses of biological macromolecules" (54).

The ion source (Figure 4) is the interface between LC and the mass spectrometer. The mobile phase flow from the LC is sprayed into the ion source via a capillary needle. High temperature and drying gas is applied in the ion source which will make the mobile phase evaporate and ions in gas phase are formed. An electrical gradient is formed between the capillary needle and the entrance to the mass analyser which will make the ions enter the mass spectrometer (55). The transformation of ions from liquid to gas phase as well as the migrations of the ions from atmospheric pressure to high vacuum, are critical steps.

One drawback of LC-MS is the occurrence of matrix effects. Matrix effects arise when the analytes of interest co-elutes with matrix components. Less volatile compounds change the droplets formation or droplets evaporation. This phenomenon will affect the amount of charged ions in the gas phase reaching the detector (56). This will lead to suppression or an enhancement of the detector response. Ion suppression has been demonstrated to be more prominent using ESI compared to APCI. Though, the choice of sample preparation will also influence the matrix effect (37, 53). There are two common approaches for studying matrix effects. The first is a post-column infusion of the analytes of interest while injecting a blank matrix sample. This will lead to a constant signal. in the detector if there are no any eluting compounds that will suppress or enhance the ionization (57). The second approach is determination and comparison of peak areas in different sample sets. Analytes spiked in neat solution, analytes spiked before extraction in blank matrix and analytes spiked after extraction in blank matrix (58). These experiments will then be used for calculation of the matrix effect as well as the recovery and process efficiency (59).

The identification of an unknown analyte is secured based on accurate retention time and mass spectral data. One advantage with mass spectrometry for quantitative bioanalysis is the possibility to use isotope labelled analogues as internal standards. Deuterated analogs have the identical chemical properties as

the analytes and will compensate for possible losses during sample preparations and/or changes in detector response. This leads to increased accuracy and precision (60).

The mass spectrometer consists of a quadropole mass filter (Figure 4), which is composed of four parallel rods having alternating voltage applied. The charged ions from the ion source are focused and transferred between the rods into the detector. The ions will be influenced by the electrical field and only ions with the correct m/z ratio will pass through and enter the detector. Ions with wrong m/z ratios will hit one of the rods due to incorrect amplitude (61).

The tandem mass spectrometry (MS/MS) consists of three quadropoles linked together. The second quadropole (Q2) will function as a collision cell. A precursor ion, often the molecular ion, is selected in Q1 and then fragmented in Q2 by applying high energy and collisions with N_2 or Ar. A fragment is than selected in Q3, called product or daughter ion, and is entering the detector. This type of monitoring is called selected reaction monitoring (SRM) (62).

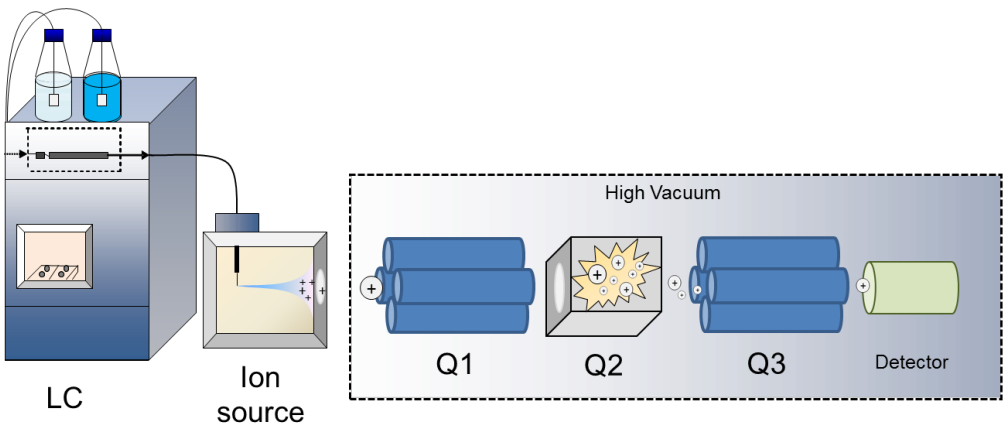


Figure 4 A LC connected to a tandem mass spectrometer, which consist of 3 qudropoles. Q1 and Q3 function as mass filters and Q2 function as a collision cell.

The more modern ultra-high performance liquid chromatography (UHPLC) is widely used since a few years ago. The pumps are designed to operate at higher pressures than conventional HPLC leading to the capability to function with

stationary phases of small sub-2µm particles. These systems results in increased separation power and reduced retention times (47).

The independent confirmation method is an important part of the drug testing strategy, confirming the screening result. SIM or SRM are generally used. To secure the selectivity two SRM transitions are monitored. The identification criteria are correct chromatographic retention time and correct relative ratio between the monitored ions (56).

In the beginning of the 1980:s a HPLC with ultra violet (UV) detection was developed for determination of morphine and metabolites in urine and plasma (3, 63). This method was important for the study of morphine pharmacokinetics (3) This method was the first to determine morphine, M3G and M6G in urine and plasma and was based on sample preparation using solid phase extraction (63).

LC was hyphenated with MS in the 1990:s (56). In the middle of the 1990:s the beginning of using LC-MS for opiate analysis in plasma and to some extent urine was introduced (46, 64, 65). Both ESI and APCI interface has been used for opiate analysis (37, 53, 66, 67).

The introduction of LC-MS/MS analysis increases the selectivity and sensitivity further. Improved selectivity will allow less sample purification. This will not necessarily promote an increase of the matrix effect ion suppression (37). The use of LC-MS/MS got more common for determination of morphine and its metabolites in plasma. The sample preparation of choice was still solid phase extractions although there were some studies regarding protein precipitations (53, 68-70). A few years later direct injection or dilute and shoot was developed for opiate analysis in urine (53, 65).

To demonstrate that bioanalytical methods are reliable and reproducible for the intended use a validation is needed. Guidelines from European Medicines Agency (EMA) (71) and the U.S Food and Drug Administration (FDA) (72) have been proposed for method validation concerning; selectivity, sensitivity, reproducibility, carry-over, calibration, accuracy and precision, dilution integrity, matrix effect and stability (59).

2 AIMS

The overall aim of this thesis was to gain additional knowledge regarding morphine and heroin metabolism by focusing on 6-acetylmorphine, morphine-3-sulfate and morphine-6-sulfates. Both bioanalytical and clinical aspects were of interest.

The specific aims were:

Study I

- Prepare reference material for morphine-3-sulfate and morphine-6-sulfate since they were not commercially or otherwise available.
- Develop an LC-MS/MS method for urine and plasma.
- Application in a preliminary study to confirm the metabolites in plasma and urine.

Study II

- Validate a routine LC-MS/MS urine drug testing method for opiates regarding reliability and biomarkers.
- To study if the number of analytes could be reduced and to evaluate how this would effect the interpretation of possible intake.

Study III

- Thoroughly investigate morphine-3-sulfate and morphine-6-sulfate and their presence and formation *in-vivo* and *in-vitro*.

Study IV

- Study the metabolic interaction of heroin metabolism.
- Study why morphine is not formed after a heroin administration/ingestion in some individuals.
- Investigate and confirm this inhibition both *in-vivo* and *in-vitro*.

3 METHODS

3.1 CLINICAL SAMPLES

3.1.1 Study I

De-coded surplus urine and plasma samples from the routine flow sent to the laboratory for analysis were used. The urine samples were selected based on the presence of the heroin metabolite 6-AM (>2 ng/ml). Ethical permit Dnr 2008/1087-32.

3.1.2 Study II

Urine samples studied were sent to laboratory for drug testing. All confirmed opiate positive urine samples during a four year time period are included (n=3155). In addition, 199 de-coded surplus urine samples from the routine flow were analyzed for method comparison with GC-MS. Ethical permit Dnr 2008/1087-32.

3.1.3 Study III

Samples were collected from 11 cancer patients treated with morphine (Dolcontin) per os or via continuous, subcutaneous infusion. From each patient one blood and urine sample was collected at the same time-point. Samples were collected twice from the same patient when possible which resulted in 13 plasma and 12 urine samples collected. Ethical permits Dnr 2010/570-32; 2012/1839-31/4.

In addition, 62 blood samples were analyzed from 21 newborns treated with morphine by continuous infusion as part of the European FP7 NeoOpioid project. A maximum of four samples with a total blood volume less than 0.8 ml were collected from an existing catheter from each patient. Ethical permit Dnr 2010/570-32.

Further 196 de-coded surplus urine samples from heroin drug addicts were analyzed. The samples were selected based on a confirmed positive result for 6-AM (>2 ng/ml). Ethical permit Dnr 2008/1087-32.

3.1.4 Study IV

De-coded surplus urine samples from the routine flow sent to the laboratory for opiate analysis. The first selection was based on a positive screening result (cut off >300 ng/ml) and the second selection was based on positive confirmation result (6-AM >2 ng/ml). A total number of 693 urine samples were evaluated. Ethical permit Dnr 2008/1087-32.

3.2 LIVER TISSUE AND CYTOSOL

3.2.1 Study III and IV

In-vitro studies were performed on human livers and one pool of fetal cytosol Ethical permits Dnr 429/01; 280/00.

3.3 SAMPLE PREPARATION PROCEDURES

3.3.1 Plasma; Study I and III

The sample preparation of plasma consisted of a protein precipitation with acetonitrile. Fifty microliter plasma with 100 µl acetonitrile containing deuturated internal standards (M-d_3, M3G-d_3 and M6G-d_3) was vortex mixed for ~10 seconds. The mixture was centrifuged for 10 min at 3000 rpm. The supernatant was evaporated with N_2 at 40 ºC until dryness and further reconstituted with 30 µl aqueous 0.1 % formic acid.

3.3.2 Urine; Study I-IV

The urine samples were diluted five-fold with water. An aliquot of 125 µl water containing deuturated internal standard was added to 25 µl urine in an autosampler vial.

3.3.3 Liver cytosol; Study III

Human liver cytosol pools was incubated with 100 µM morphine in TRIS HCl buffer (0.05 M with 0.25 mM $MgCl_2$) pH 7.4 and 0.05 M PAPS. The incubation time were 25 min at 37 ºC the total volume were 125 µl. The reaction was stopped by adding 125 µl ice-cold acetonitrile. The supernatant was removed and stored at -20 ºC prior analysis after centrifugation at 4000 × g for 15 min at 4 ºC.

3.3.4 Liver homogenate; Study IV

Pieces of human liver tissues were homogenized in 0.05 M TRIS-HCl buffer, pH ~7.5. A volume of 10 µl liver homogenate, 0.385 mg/mL protein equivalent and TRIS-HCL buffer was mixed with either ~4 µl of; acetylcodeine, acetyl salicylic acid, caffeine, cocaine, compound 3, compound 4 EtOH, lidocaine, loperamide or procaine with concentrations between 6.1-61 µM. This mixture (total volume of 0.2 ml) was pre-incubated at 37 ºC for 5 min. Further 4 µl of 6-AM solution (6.1 µM) was added and the incubation continued for 15 min at 37 ºC. The reaction was stopped by adding 200 µl ice-cold acetonitrile and placing the test tubes on ice. The internal standard, codeine-d_3, was added (10 µl) together with 10 µl of the sample solution and 80 µl 0.1 % aqueous formic acid in an autosampler vial prior analysis.

3.4 BIOANALYSIS

3.4.1 LC-MS/MS Study I-IV

Quantification of opiates was performed with LC-MS/MS. The LC system consisted of an AQUITY UPLC system connected to a Quattro Premiere XE or a XEVO TQ mass spectrometer (Waters, Milford, MA, USA). The tandem mass spectrometer was operated in positive electrospray mode using selected reaction monitoring (SRM). The specific transitions monitored are presented in each paper. Separation was achieved with reversed phase chromatography using an AQUITY UPLC HSS T3 2.1×100 mm, 1.8 µm or an AQUITY UPLC BEH C18 2.1×100 mm, 1.7 µm. The mobile phase A consisted of a 0.1 %; aqueous formic acid and mobile phase B; methanol or acetonitrile. Gradient elution was used

with a flow rate of 0.2 ml/min or 0.35 ml/min. The analytical column was always kept at 60 ºC. Different chromatographic systems were developed to obtain optimal retention and separation for the analytes of interest.

3.4.2 LC-HRMS

The LC system consisted of a Dionex Ultima 3000 coupled to a Thermo Scientific Q Exactive mass spectrometer (Fremont, CA, USA) operating in positive mode, full scan ranged within 90-1,350 m/z and a 70 000 resolution power. Separation was achieved on an AQUITY UPLC HSS T3 2.1×100 mm, 1.8 µm with mobile phase consisted of 2 mM ammonium formate and 0.2 % ammonia solution (25 %). Mobile phase B consisted of 100 % methanol with the same amount of ammonium formate and ammonia. The column was kept at 50 ºC and the flow rate was 0.3 ml/min with a total run time of 18 min.

3.4.3 CEDIA immunoassay for opiates

The screening assay was applied on an Olympus AU 640 (Beckman Coulter, Indianapolis, IN, USA) using CEDIA opiate reagents (Thermo Fisher Scientific, Waltham, MA, USA). Cut off at 300 ng/ml and the measuring range from 0-2000 ng/ml, 5.4 % CV at 390 ng/ml (n=212) and 6.7 % CV at 190 ng/ml (n=214).

3.4.4 DRI Ethyl Glucuronide and Ethyl Alcohol

The screening assay for Ethyl glucuronide (EtG) and Ethyl alcohol (EtOH) were performed on an Olympus AU 640 using DRI enzyme EtOH enzyme assay and DRI EtG immunoassay from Thermo Fisher Scientific. The cut off for EtG is 500 ng/ml and 5 mM for EtOH. The measuring range for EtG is 0-2.0 µg/ml, 4.5 % CV at 0.375 µg/ml (n=211) and 3.2 % CV at 0.625 µg/ml (n=209). The measuring range for EtOH is 0-20.83, 6.1 % CV (n=210) at 2.55 mM and 4.2 % CV (n=210) at 7.5 mM.

3.4.5 GC-MS for opiates

The GC-MS system used was a Thermo Finnigan Voyager Toxlab system (Thermo Electron Co, Waltham, MA, USA). The mass spectrometer was operated in the electron ionization mode using selected ion monitoring (73). The column used was a J&W DB-1701(30 m x 0.25 mm x 0.25 film thickness) (Agilent Technologies Inc., St. Clara, CA, USA). The carrier gas used was He. The total run time was approximately 20 min. The sample preparation consisted of hydrolysis by hydrochloric acid, automated solid phase extraction using Bond Elut Certify LRC 130mg from Agilent Technologies and formation of silyl derivatives. The cut off was 150 ng/ml for total morphine and codeine. For 6-AM the cut off was 10 ng/ml. For analysis of 6-AM the hydrolysis step was omitted. The inter assay imprecision (11) was below 10 %.

4 RESULTS

4.1 STUDY I

Synthesis and bioanalytical evaluation of morphine-3-O-sulfate and morphine-6-O-sulfate in human urine and plasma using LC-MS/MS

4.1.1 Synthesis of M3S and M6S

A new synthetic route was developed for synthesis of M6S and M3S.

When following earlier reported procedures the product of M6S was impure as was revealed by careful LC-MS analysis. The resulting product was contaminated with residues of morphine. This observation was made when studying the intermediate product 3-acetylmorphine (3-AM). The acetylation process by Welsh resulted in 3-AM containing both the side-product heroin as well as unreacted morphine (74). In addition the purified 3-AM was unstable leading to degradation within days during dark and cold storage (-20 ºC) in the dark. This resulted in a mixture containing 3-AM, heroin and morphine.

Regarding M3S the problem was obtaining the intermediate 6-AM in pure form. Earlier published procedures had to be improved. This was done by using a protective silyl group at the 6-position.

The value of using careful LC-MS analysis for product characterization was demonstrated in this work. For example, the characterization of the purity of the intermediate product morphine-3-acetat-6-sulfat became of importance due to resulting in a final M6S pure product. Several batches contained impurities of residual, heroin and 6-AM (Figure 5).

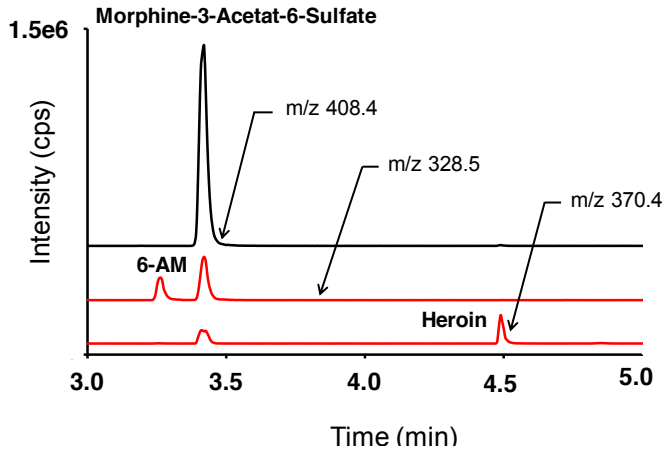


Figure 5 A chromatogram during characterisation of the intermediate morphine-3-acetat-6-sulfat batch 1878 with LC-MS using selected ion monitoring. This batch also contained impurities of 6-AM and heroin.

These findings led to new developed procedures for the synthesis of M3S and M6S as dihydrates. Resulting in a high product purity >99.5 for M6S with an overall yield of 41 %. For M3S the purity was >98 % and an overall yield of 39 %. In order to ascertain the correct product identity single X-ray analysis was used.

4.1.2 Method development and validation

Different chromatographic systems were evaluated resulting in using an ACQUITY HSS T3 2.1×100 mm, 1.8 µM with mobile phase A containing 0.1 % aqueous formic acid and mobile phase B consisting of methanol. The chromatography selected was based on separation between M3S and M6S and the separation compared to the other morphine metabolites; M3G, M6G and morphine itself. Only one SRM transition was usable for the morphine sulfates and they were the same. For that reason identification was established by monitoring the analytes also in negative mode. Morphine-d_3 was chosen as the internal standard for both sulfates

The measuring range for plasma was 5-500 ng/ml for M3S and 4.5-454 ng/ml for M6S. In urine the measuring range was 50-5000 ng/ml for M3S and for M6S 45.4-4544 ng/ml. The response was linear in the measuring ranges. In Figure 6 a chromatogram of a urine calibrator is shown. The intra-assay and total imprecision had CV:s less than11 % with accuracy between 98-111 % for both analytes in urine and plasma. The matrix effect was of significance for both M3S and M6S in plasma, showing an average suppression of the signal of 37 % for M6S and 48 % for M3S. In urine, the suppression of the signal was <15 % for both analytes.

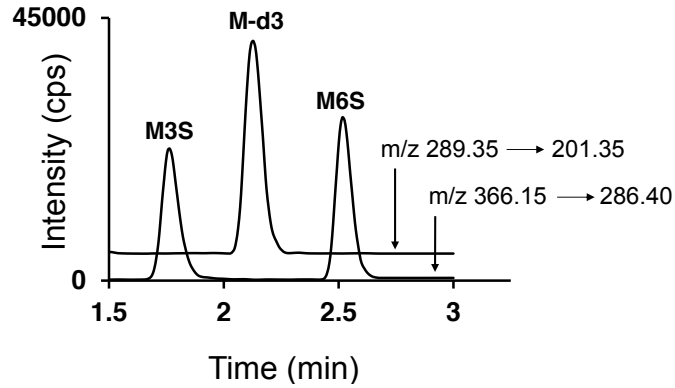


Figure 6 A chromatogram of a urine calibrator containing 500 ng/ml M3S and 454 ng/ml M6S.

4.1.3 Application

Nine plasma samples were obtained from patient receiving morphine and 8 of the samples contained 5.8-12.9 ng/ml of M3S. In the 18 urine samples, probably from heroin intake, all contained detectable M3S and 13 samples quantifiable concentrations between 69-1500 ng/ml. No urine or plasma sample contained detectable concentrations of M6S. The LOD for M6S was 4 ng/ml in urine and 0.3 ng/ml in plasma. In Table 2 the results are summarized.

Table 2 M3S and M6S concentrations for the investigated patient samples

Study	M3S		M6S	
	n	ng/ml	n	ng/ml
Plasma samples (n=9)	8	6-13	0	-
Urine samples (n=18)	18	70-1500	0	-

4.2 STUDY II

Direct and efficient liquid chromatographic tandem mass spectrometric method for opiates in urine drug testing –Importance of 6-acetylmorphine and reduction of analytes

4.2.1 Method design and validation

The method was designed according to our previous work (75) but modified using Waters UPLC system and a more modern mass spectrometer.

The measuring range for morphine and codeine was 150-1 000 000 ng/ml for M3G 150-600 000 ng/ml, for codeine glucuronide (CG) 150-400 000 ng/ml, for M6G 150-50 000 ng/ml and for 6-AM 2-30 000 ng/ml. The CV values for total imprecision were less than 16 % and the accuracy was within 96-106 %. External quality controls from the proficiency program College of American Pathologists showed good agreement for total morphine, codeine and 6-AM with an accuracy within 87-110 %. The matrix effect was extensive for the first eluting compound M3G, displaying an average suppression of the signal of 78 % in the addition experiment. When investigating the matrix effect with an infusion experiment M3G will elute during the recovery of the signal (Figure 7).

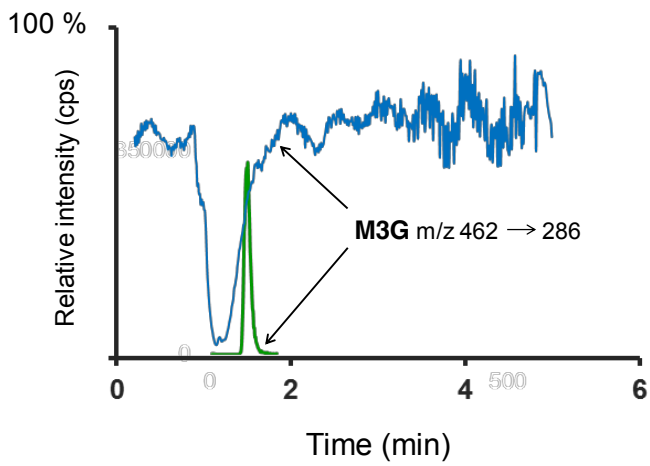


Figure 7 Blue line displays a chromatogram of infusing M3G (9500 ng/ml) at 10 µl/min while injecting a blank urine sample monitoring the SRM transition m/z 462 to 286. Green line is a chromatogram of M3G eluting at 1.4 min.

The identification was based on correct relative retention times and ion ratio for two SRM products. Figure 8 shows an example chromatogram obtained from a sample collected after a heroin intake.

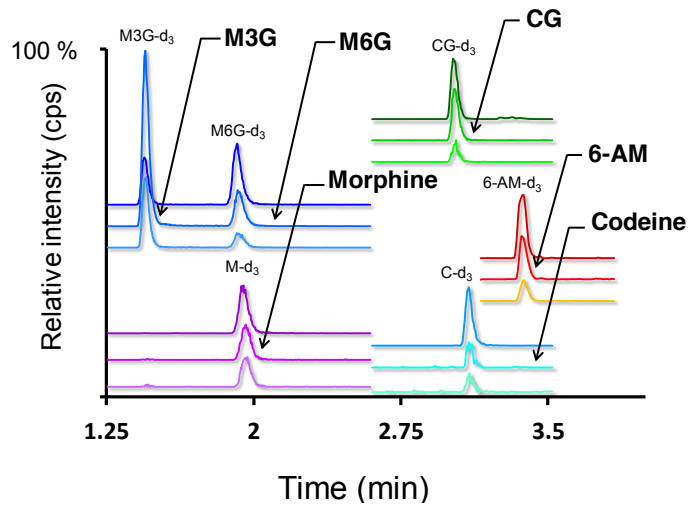


Figure 8 A chromatogram after a heroin intake containing 14 ng/ml 6-AM, 4590 ng/ml M3G, 965 ng/ml M6G, 340 ng/ml morphine, 225 ng/ml CG. Codeine was below cutoff.

The qualitative method comparison with the reference GC-MS method showed good agreement. A number of 199 urine sample which were found positive in CEDIA opiate screening were analyzed with the GC-MS method and the LC-MS/MS method. Comparison for total morphine and codeine is shown in Figure 9. For total morphine only two samples deviated and contained concentrations close to the cut off. For total codeine one single sample deviated which were a false positive sample. The deviating results were within the uncertainty of the measurement.

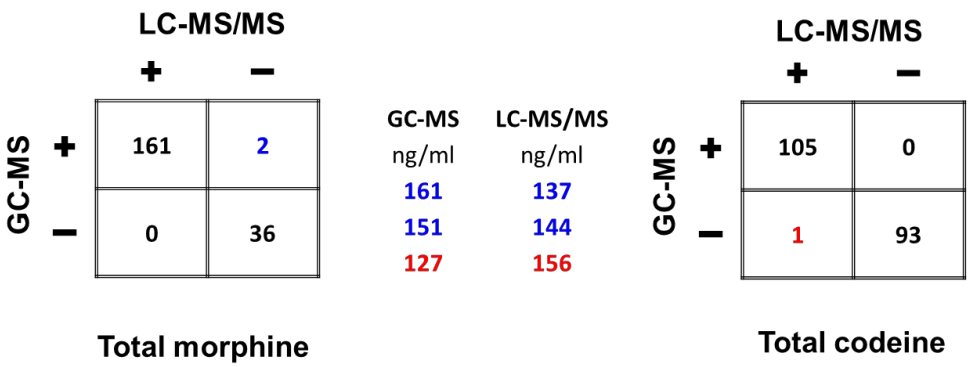


Figure 9 Method comparison using GC-MS as a reference method (n=199) showed 2 false negative samples for total morphine and one false positive for total codeine.

4.2.2 Interpretation strategy

After this method had been in routine use for 3 years an evaluation of data was performed. The evaluation was made by using our data pool containing 3155 samples (Figure 10) plotted according to total morphine and total codeine content. Total morphine consists of M3G, M6G and morphine. Total codeine consists of codeine and CG. Four clusters are revealed in the plot. Two containing only total morphine or codeine and the other two contains different proportions of morphine and codeine.

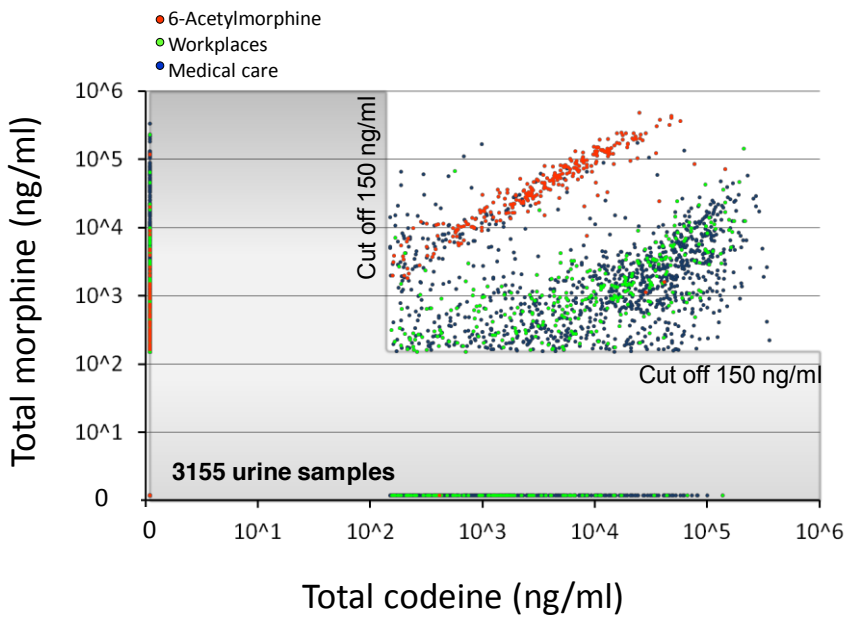


Figure 10 Results from 3155 authentic urine samples. Red dots are samples containing 6-AM, Blue dots represent samples from the medical care and green samples are from workplaces all are 6-AM negative. Values below cut off are depicted as zero.

4.2.2.1 Heroin intake

Expected analytes after a heroin intake is mainly morphine and its metabolites, but also 6-AM, codeine and CG. A safe criterion for a heroin intake is presence of the heroin metabolite 6-AM. A number of 365 samples contained 6-AM. Of these there were 95 samples (26 %) that contained total morphine <2500 ng/ml. A heroin intake can also be defined by a ratio of total morphine over codeine without presence of 6-AM. The samples containing 6-AM and quantifiable concentrations of total morphine and codeine were used as reference to calculate a 95 % prediction interval. The frequency diagram is displayed in Figure 11 of the ratio total morphine over codeine for the samples containing 6-AM. The prediction interval was calculated to be 4.4-28.4.

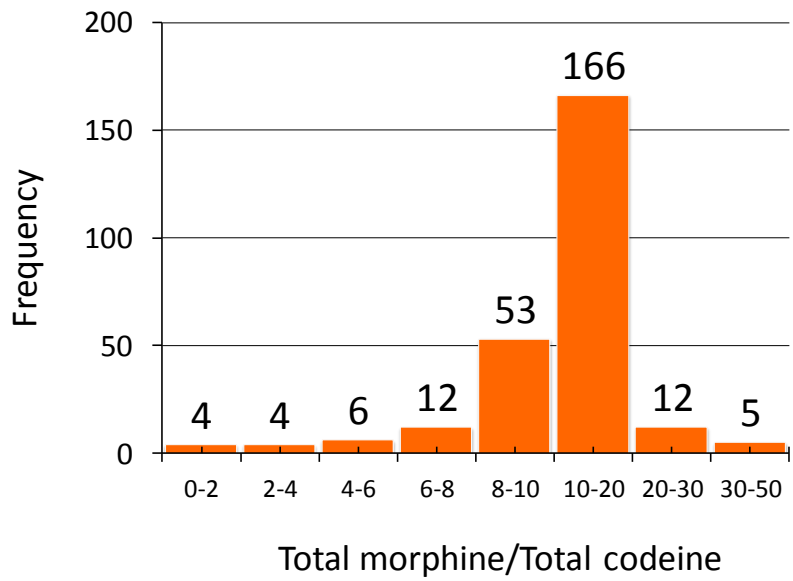


Figure 11 A frequency diagram for samples containing 6-AM and quantifiable concentrations of total morphine over codeine (n=262). The mean ratio is 11.5 and a 95 % prediction interval is calculated to be 4.4-28 for total morphine >2500 ng/ml.

When applying the ratio interval and the 6-AM criterion we get a total number of 444 samples. An addition of 78 samples fulfils the ratio interval criterion (Figure 12).

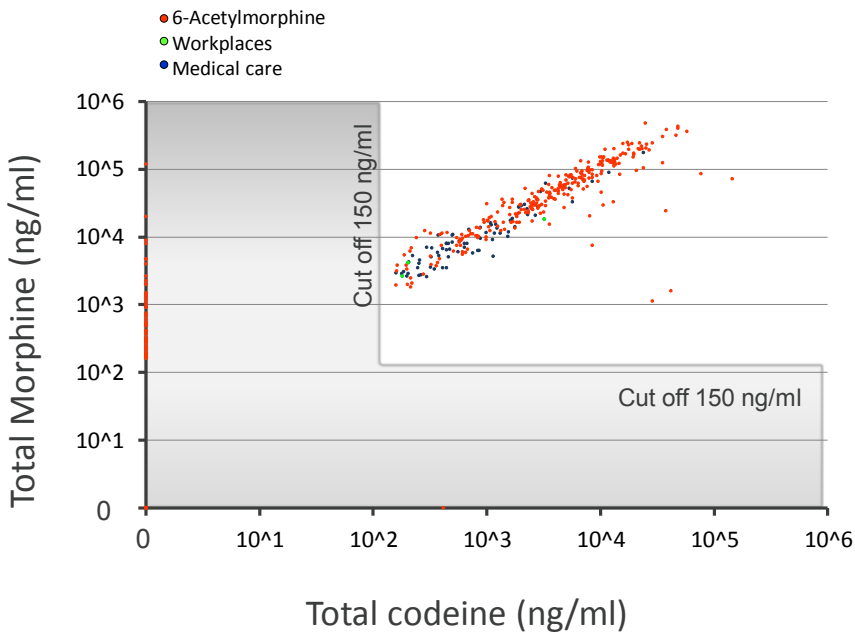


Figure 12 Samples that fulfil the heroin criteria's. Red dots are samples containing 6-AM and blue and green dots are samples which fulfils the total morphine/codeine ratio 4.4-28.

4.2.2.2 Poppy seed

Low concentrations of morphine may originate from dietary intake of poppy seed due to this reason a lower limit must be set. In this study the lower limit was set to 2500 ng/ml for total morphine. Though a codeine intake may also lead to low concentrations of total morphine therefore samples with a ratio >2 for total morphine over codeine must be fulfilled. Thus a poppy seed intake in our study is defined as total morphine < 2500 ng/ml with a ratio >2 for total morphine over codeine.

4.2.2.3 Codeine

For setting a criterion for a codeine intake we used the samples from workplaces as a reference population to calculate a 95 % prediction interval (coloured green in Figure 10). The interval was calculated to be 0.009-2.58 for a codeine intake. Another criterion for a codeine intake was samples that only contained codeine. Consequently a codeine intake in our study is defined as ratio <2.58 for total morphine over codeine or samples that contain only codeine.

4.2.2.4 Morphine

Samples that contain only total morphine above 2500 ng/ml and no detectable codeine or 6-AM is interpreted as a possible morphine intake or possible heroin intake.

4.2.2.5 Proposed interpretation strategy

We have proposed a possible interpretation strategy on the basis of statistical calculations. The interpretation strategy is outlined in Figure 13 and is categorizing all urine samples in our data pool.

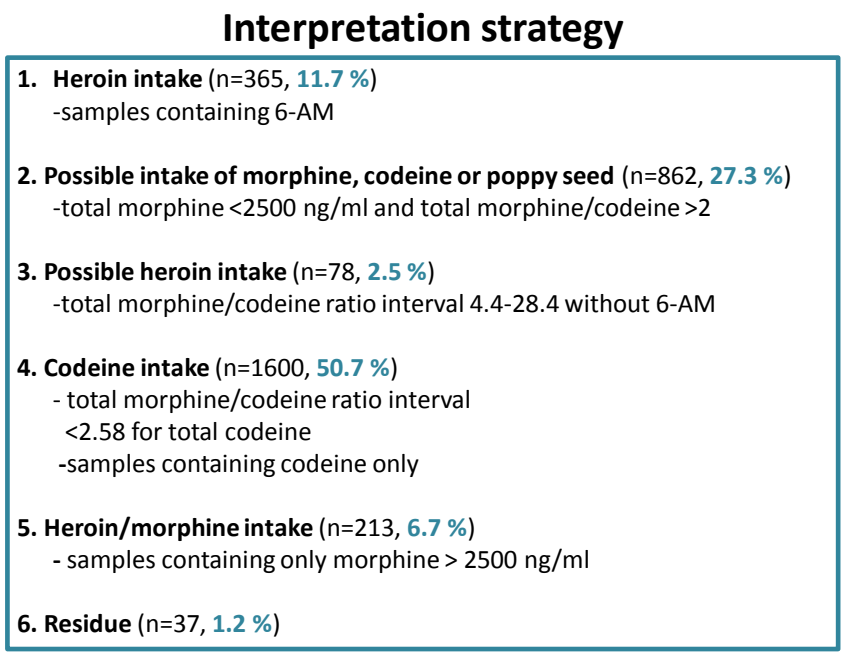
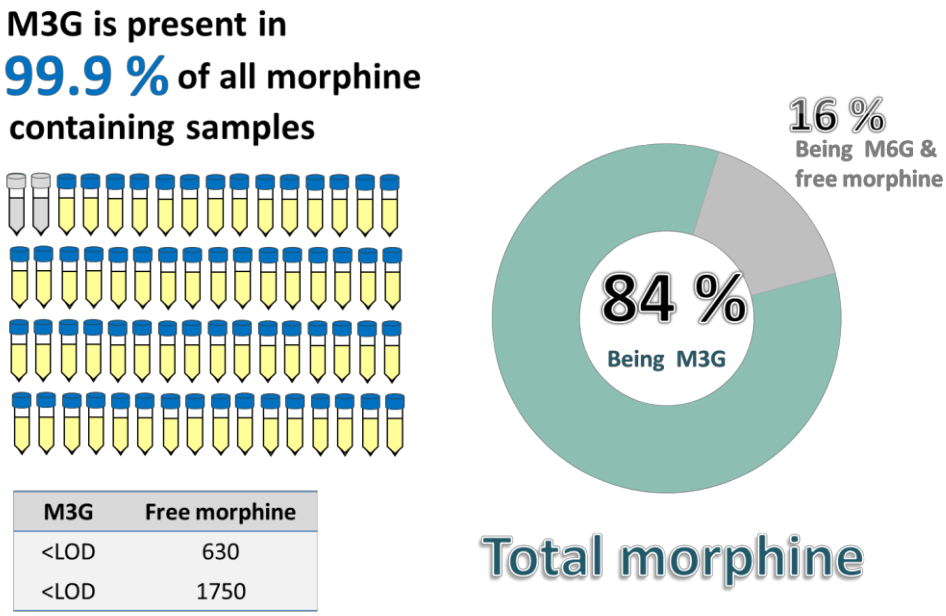


Figure 13 Proposed interpretation strategy

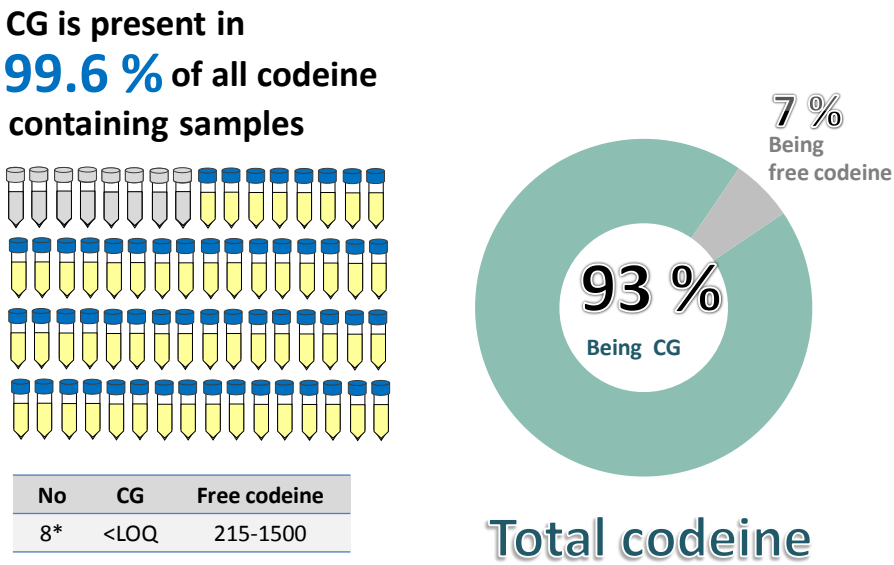
4.2.3 Reduction of analytes

The possibility to reduce the number of analytes in routine testing was also evaluated. The main component for the total morphine was M3G and for total codeine it was CG. M3G was present in 99.9 % in all of the morphine containing samples (n=2674). The fraction of M3G of total morphine was 84% ±14 (SD). CG was present in 99.6 % of the codeine containing samples (n=2021), with 94% ±14 (SD) of total codeine being CG. Figure 14a-b displays M3G and CG in relations to total morphine and codeine in graphics.



M3G	Free morphine
<LOD	630
<LOD	1750

Figure 14a M3G was present in 99.9 % in all of the morphine containing samples (n=2674). The fraction of M3G of total morphine was 84% ±14 (SD)



No	CG	Free codeine
8*	<LOQ	215-1500

Figure 14b CG was present in 99.6 % in all of the codeine containing samples (n=2021). The fraction of CG of total codeine was 93% ±12 (SD)

The limits used in the interpretation strategy given in Figure13 were adjusted. When this was applied for interpretation of the data set it gave a categorization agreement of 98.7 %.

4.3 STUDY III

On the presence of morphine-3-sulfate and morphine-6-sulfate in human urine and plasma, and formation in liver cytosol

4.3.1 Method development

Pronounced ion suppression for the first eluting compounds M3G and M3S in previous studies lead to investigations with "straight phase" chromatography. Also a new filtration technique for removal of phospholipids from plasma was tested. Unfortunately the results were not satisfying because the matrix effects were not much reduced, see Table 4. The retention order of the analytes did change using straight phase chromatography (SP) (Figure 15). However, this led us to go back to the initial reversed phase chromatography (RP) method. This work was presented as a poster at the IATDMCT conference in Salt Lake City, 2013.

Table 3 Validation of the matrix effect by addition experiments comparing different sample preparation and chromatography systems.

	M3S		M6S		M		M6G		M3G	
	SP	RP	SP	RP	SP	RP	SP	RP	SP	RP
PPT	-17	-67	-16	-24	-92	7	377	-12	376	-79
Ostro	-38	-37	-52	-18	-90	9	185	1	239	-76
Phree	-29	-33	-50	-5	-88	25	389	7	185	-80

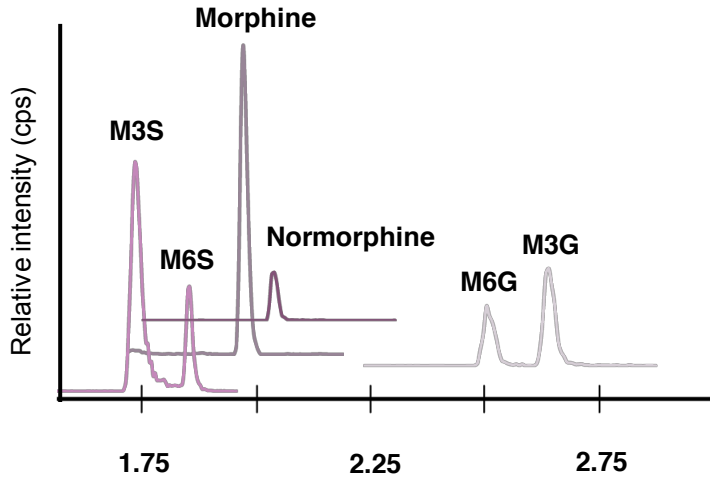


Figure 15 A chromatogram using straight phase chromatography with an ACQUITY BEH amide column 2.1x10 mm, 1.7 µm. The mobile phases consisted of 5 mM ammonium formate adjusted with formic acid to pH 3 and acetonitrile at a flow rate of 0.6 ml/min. The total time of analysis was 3.5

4.3.2 Formation of M3S and M6S In Vitro

Both M3S and M6S were formed, *in-vitro* (Figure 16). Only a small portion of morphine was converted to sulphates 0.0041 % of M3S and 0.010 % of M6S. The formation of the sulfated morphine metabolites was seen in both adult and fetal liver cytosol. The formation rate of M3S was 0.17 nmol/mg protein per minute (n=8) and for M6S the formation rate was 0.43 nmol/mg protein per minute (n=6) in the adult liver cytosol using a substrate concentration of 100 µM. As a control, incubation was also performed with PAPS and morphine but

without cytosol. No sulfated morphine metabolites were found.

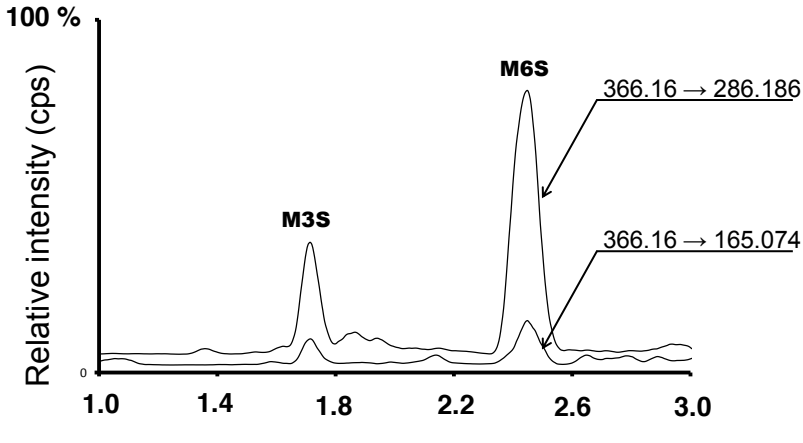


Figure16 A Chromatogram obtained of a morphine incubation with adult liver cytosol containing 7.6 nM M3S and 20 nM M6S, substrate concentration of 100 μM.

4.3.3 Presence of M3S and M6S *in-vivo*

M3S was reconfirmed as a morphine metabolite in both urine and plasma in the *in-vivo* studies, see Table 5. As expected M3S concentrations are higher in the urine samples than for the plasma samples. In plasma M3S concentrations were in the range of 8-9 ng/ml. The M3S/M3G ratio differs significantly between the cancer patients and the newborn patients. For the newborn patients the median ratio was 30 times higher than for the cancer patients.

The identfication of M6S as a morphine metabolite *in-vivo* was not as convincing. Only one plasma sample and one urine sample for M6S were detected. One plasma sample from the newborns study contained 7 ng/ml M6S. In the urine sample from the drug testing population M6S was detected at a

concentration of 9 ng/ml. The identification M6S was not convincing at these low concentrations since the qualifier ion intensities were weak.

Tabel 4 A summary of M3S in the *in-vivo* studies.

Study	M3S			M3S/M3G		
	n	ng/ml	median	n	%	median
Cancer patients plasma (n=13)	6	5-54	8	6	0.29-0.54	0.41
Newborn patients plasma (n=62)	17	5-21	9	17	2.66-24.1	12.7
Cancer patients urine (n=12)	9	53-2920	180	9	0.84-3.38	1.39
Drug testing urine (n=196)	88	52-1780	100	88	0.10-2.44	0.2

4.4 STUDY IV

Studies on the inhibition of heroin metabolism

4.4.1 Characterization of atypical samples

The number urine samples in the data set that contained 6-AM was 693. Defining atypical samples by 6-AM >2ng/ml and total morphine <1000 ng/ml resulted in 125 samples (18 %). If using a more strict criterion with 6-AM >10 ng/ml and total morphine <300 ng/ml the number of atypical samples was 80 (11.5 %). When plotting a frequency diagram of the ratio 6-AM/total morphine a bimodal distribution was revealed, see Figure 17. Atypical samples were then defined as 6-AM/total morphine ration >0.26 ending up with 78 atypical samples (11.2 %).

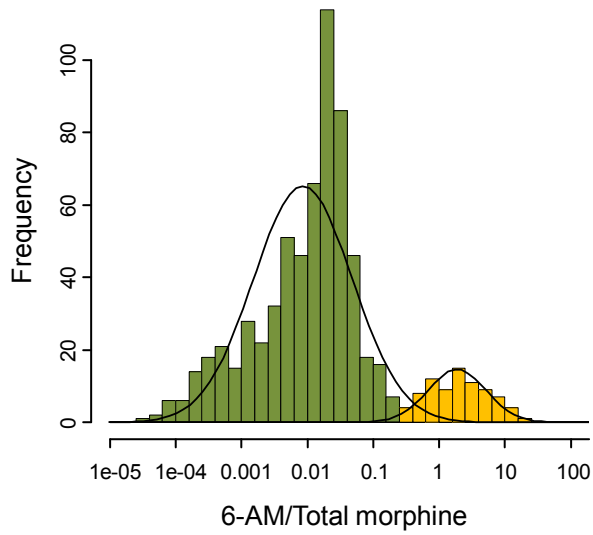


Figure 17 Frequency plot over the 693 positive heroin samples. The 78 atypical samples are displayed in yellow bars with a ratio >0.26.

4.4.2 Study of 6-AM deacetylation *in-vitro*

The *in-vitro* system that tested the activity of human liver homogenates provided an 86 % mean conversion rate of 6-AM to morphine displaying a large inter variability for the ten individuals CV=52 % (n=30). The strongest inhibition effect was seen for compound 3, compound 4 and loperamide. The *in-vitro* inhibition of morphine in the presence of each substrate is shown in Figure 18. For some "substrate" there was a large intervariability for the inhibition effect. A diagram is shown in Figure 19 displaying the inhibition for each "individual" when caffeine is being present in the incubation system. For the liver tissue 76 we got an inhibitory effect of 68 % but for the liver tissue 52 we only got 2 % inhibition.

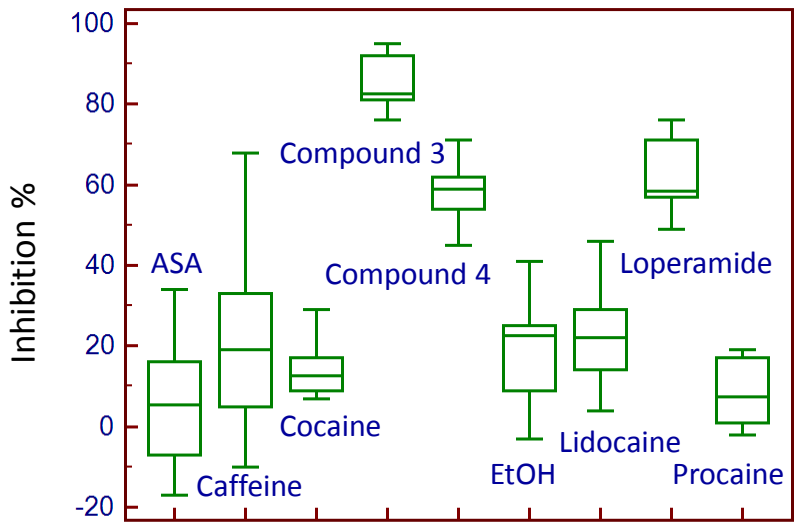


Figure 18 *In-vitro* inhibition of morphine from 6-AM during presence of substrate. The strongest inhibition effect was seen for compound 3 (median= 83), compound 4 (median= 59) and loperamide (median= 59).

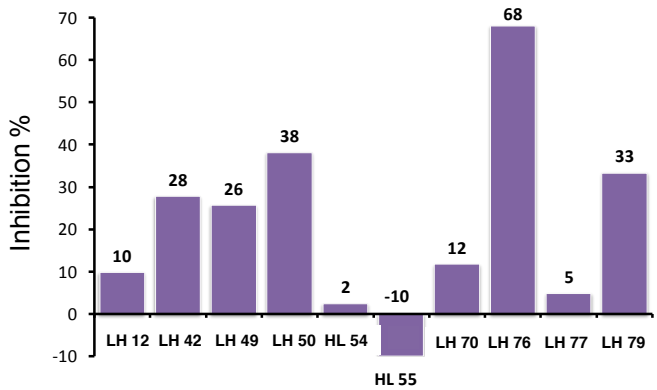


Figure 19 A diagram of the inhibitory effect of caffeine during the *in-vitro* conversion of 6-AM to morphine in ten individual liver tissues.

4.4.3 Search for inhibitors in authentic samples

An investigation was made to find possible inhibitors in authentic samples using different sample sets. Eighty patient samples which contained a ratio 6-AM/total morphine <0.26 ng/ml were screened for EtG and EtOH. EtG was present in 40 % of the samples and EtOH was present in 5 % of the samples. In the samples with a ratio 6-AM/total morphine >0.26 ng/ml (n=35) EtG was present in 34 % of the samples and EtOH was present in 14 % of the samples. In another set of samples consisting of 40 atypical (6-AM >2ng/ml and total morphine <1000 ng) and 40 normal samples (6-AM >2ng/ml and total morphine >1000 ng) screening was performed with LC-MS/MS for five possible inhibitors; salicylic acid, cocaine, lidocaine, loperamide, procaine and heroin. Very few samples were above cutoff >1 µM (salicylic acid >750 ng/ml). Only salicylic acid was more pronounced in the atypical group for six samples.

When searching for compound 3 detectable concentrations (< 1 ng/ml) were found in almost every sample (n=44). There was a slightly higher mean peak area in the atypical samples. Compound 4 was not detected in any of the samples. The metabolite from compound 3, ATM3 and the metabolite for compound 4 ATM4 co-eluted but the peak areas were higher in the atypical samples.

Forty-four samples were investigated using a non-targeted metabolomic approach with LC-HRMS. The samples were selected by a more strict criterion, categorizing the atypical group with a ratio 6AM/total morphine > 0.26, n=19. In the normal group a total of 25 samples were selected based on a ratio 6AM/total morphine <0.26.

The samples were processed with the Sieve software program. Most of the components were more abundant in the “normal” group. As expected a significantly higher abundance for morphine-3-glucuronide and morphine-6-glucuronide were seen in the ”normal” group. No obvious candidates for having enzyme inhibition effects were revealed from the data analysis.

5 DISCUSSION

This was the first study using mass spectrometry to study the presence of the conjugated sulphate morphine metabolites M3S and M6S. It was demonstrated that both M3S and M6S can form when studied *in-vitro,* but in small amounts. The *in-vivo* studies resulted in convincing evidence of M3S as a morphine metabolite in both urine and plasma using the criteria of retention time and monitoring two SRM transitions. However, there was only weak evidence that M6S actually forms *in-vivo* since only two samples contained M6S. The identification was not as convincing due to weak abundance of the qualifier transition. In earlier reported studies from Yeh and co-workers when determining M3S and (M6S) there were uncertainties in the analytical technique used (27, 28) since the selectivity using TLC is rather low. However, the Yeh studies proposed a formation of 1-5 % for M3Srelative to M3G in urine. This is in good agreement with our results. Since only small amounts of the sulfates are present in urine and plasma compared with the glucuronides it may be concluded that they can be neglected in most respects. Maybe M3S shold be considered when doing validation concerning possible interference in analytical methods for determination of morphine.

The LC-MS/MS method for urine drug testing using direct injection has been demonstrated to be accurate and reliable in routine use. A method comparison against GC-MS, which is the golden standard technique in urine drug testing, was established (ref GC-MS methods). We verified a good agreement between the techniques when using the GC-MS method as a reference method. The possibilities using LC-MS/MS in urine drug testing is to have the option to simplify the sample preparation omitting the hydrolysis and derivatization and in some cases extraction (53).

When omitting sample preparation and only dilute the urine matrix effect may be more pronounced (46). Since our method displayed ion suppression for the earlier metabolites M3G and morphine one might consider additional retention of M3G.

The aim to further simplify the analytical method by decreasing the number of analytes resulted in outlining a possible interpretation strategy. During evaluation of the data set we could display that a safe and reliable criterion for a heroin intake is to use 6-AM as a biomarker. 6-AM seems to be detected for a longer time in urine (46). We could detect 6-AM in 82 % (n=443) of all cases that could be attributed to a heroin intake displaying 6-AM as a sensitive biomarker.

We have demonstrated that there is a possibility to reduce the number of analytes to further simplify the method. When decreasing the number of analytes to the main metabolites of morphine and codeine, M3G and CG. There were only a small fraction of samples containing free morphine and codeine.

When the first developed method for M3S and M6S displayed ion suppression experiments were performed regarding changing the chromatography. Investigations were performed using different HILIC columns to retain the more hydrophilic morphine metabolites. As expected the hydrophilic M3G got the strongest retention though M3S still displayed a short retention on the column. The ion suppression did not improve significantly and for some analytes we got ion enhancement instead. Altogether we chose to continue with the original reversed phase chromatography.

We could confirm the finding in earlier reports (41-45) about the existence of the atypical metabolite pattern after a heroin intake in some individuals. Euler and co-workers suggested that the inhibition could be attributed to high concentrations of heroin and/or 6-AM resulting in inhibition of the UGT2B7 enzyme capacity or the CES:s enzyme. Another suggestion is that a heroin intake has occurred immediately after sampling. The third proposal refers to xenobiotics administered together with heroin or some unknown heroin constituent which will cause the inhibition of 6-AM (41). Our *in-vitro* studies displayed an inhibition of the conversion of 6-AM to morphine. This supported our initial hypothesis that the inhibition of carboxyesterase is caused by a substance ingested together with heroin. Regarding heroin constituents the rearrangement product from thebaine compound 3 showed a large inhibitory

effect (85 %) but only small amounts were found in the urine samples. Our conclusion is that it is likely that several substances contribute to the atypical pattern of heroin metabolism.

To summarize, in this thesis we have developed a new method for detection of morphine sulphate metabolites thus enabling the monitoring of these metabolites in human samples. In addition, a validated routine LC-MS/MS method for urine drug testing of opiates has been developed and that 6-AM is a good and sufficient biomarker for heroin.

6 CONCLUSION

The following main conclusions can be drawn

- Reference material for M3S and M6S has successfully been developed and are now accessible. For the first time an analytical method for quantification of M3S and M6S in urine and plasma have been developed with mass spectrometry. The formation of M3S and M6S are small both *in-vivo* and *in-vitro* nevertheless we have demonstrated that both M3S and M6S are morphine metabolites.

- A validated routine LC-MS/MS method for urine drug testing of opiates has been evaluated. The evaluation displayed that the 6-AM biomarker is a good and dependable criterion for a heroin intake. We have also demonstrated that this method can be reduced regarding number of analytes.

- We have reconfirmed and demonstrated the atypical pattern after a heroin intake. The phenomenon of 6-AM inhibition seems to depend on several causes.

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